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# Oral Ingestion of Collagen Hydrolysate Leads to the Transportation of Highly Concentrated Gly-Pro-Hyp and Its Hydrolyzed Form of Pro-Hyp into the Bloodstream and Skin

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**ABSTRACT:** Collagen hydrolysate is a well-known dietary supplement for the treatment of skin aging; however, its mode of action remains unknown. Previous studies have shown that the oral ingestion of collagen hydrolysate leads to elevated levels of collagen-derived peptides in the blood, but whether these peptides reach the skin remains unclear. Here, we analyzed the plasma concentration of collagen-derived peptides after ingestion of high tripeptide containing collagen hydrolysate in humans. We identified 17 types of collagen-derived peptides transiently, with a particular enrichment in Gly-Pro-Hyp. This was also observed using an in vivo mouse model in the plasma and skin, albeit with a higher enrichment of Pro-Hyp in the skin. Interestingly, this Pro-Hyp enrichment in the skin was derived from Gly-Pro-Hyp hydrolysis, as the administration of pure Gly-Pro-Hyp peptide led to similar results. Therefore, we propose that functional peptides can be transferred to the skin by dietary supplements of collagen.

**KEYWORDS:** *collagen, Gly-Pro-Hyp, Pro-Hyp, peptide, kinetics, dietary supplement*

## INTRODUCTION

Collagen is the main structural protein of connective tissues in animals. Comprising approximately 30% of all proteins in the body, it is present in fibrous tissues, such as tendons and ligaments, as well as in the cornea, cartilage, bones, skin, and blood vessels.[1] Collagen has a unique triple-helix structure with a repeated amino acid sequence of $(Gly\text{-}X\text{-}Y)_n$, in which X and Y are typically Pro and Hyp. It can be found in various food sources added as a denatured form, gelatin, or, alternatively, manufactured as dietary supplements in an enzymatically hydrolyzed form, collagen hydrolysate. Due to the high solubility of collagen hydrolysate, it can be used in drinks and as a jelly-like form, making it ideal as a food supplement and for cosmeceutical skincare.

Over recent years, various beneficial effects have been reported on the consumption of collagen hydrolysates that includes improvements in joint pains,[2] wound healing,[3,4] blood pressure,[5] and glucose tolerance,[6] as well as the moisture, elasticity, and wrinkles of facial skin[7] and epidermal barrier function.[8] Although the mechanism of most of these effects remains unknown, several studies have observed a transient increase in collagen-derived peptides, especially of Pro-Hyp,[9−11] in the blood[12−14] and skin[15] after ingestion of collagen hydrolysate. In dermal fibroblasts, Pro-Hyp has been shown to stimulate cell proliferation, increase synthesis of hyaluronic acid,[16,17] and accelerate cell migration in the skin.[18] Together, these suggest that Pro-Hyp is likely to be a key collagen-derived peptide. However, whether other collagen-derived peptides are beneficial and can be incorporated into the skin remains unclear.

Recent advancements in methods for collagen hydrolysate preparation led to the production of high tripeptide containing collagen hydrolysate (HTC-col), which has high concentrations of tripeptides comprising the Gly-X-Y sequence. This has received attention as a potential alternative to traditional food supplements of collagen due to reports of HTC-col having inhibitory effects on atherosclerosis development in hypercholesterolemic rabbits,[19] anti-photoaging properties,[20] and improvements in the dryness of the skin.[21] This suggests that collagen-derived tripeptides may also have beneficial effects on the body. Indeed, Gly-Pro-Hyp has been reported to have activity on the central nervous system after intraventricular administration[22] and also inhibits dipeptidyl peptidase-4 in vitro, which has been implicated in diabetes.[23]

Previous studies have shown that after ingestion of collagen hydrolysate, Pro-Hyp is the most enriched peptide in the blood[11] and skin.[15] In this study we tested, using HTC-col, which contains high levels of tripeptides, whether the compositions of collagen-derived peptides incorporated into the blood and skin are altered.

## MATERIALS AND METHODS

**Reagents.** Two kinds of collagen hydrolysates, HACP-01 and CPAH-5 (provided by Jellice, Miyagi, Japan), both made from porcine skin collagen by enzymatic hydrolysis, were used. HACP-01 contained mainly tripeptides (Gly-X-Y) and had an average molecular weight of

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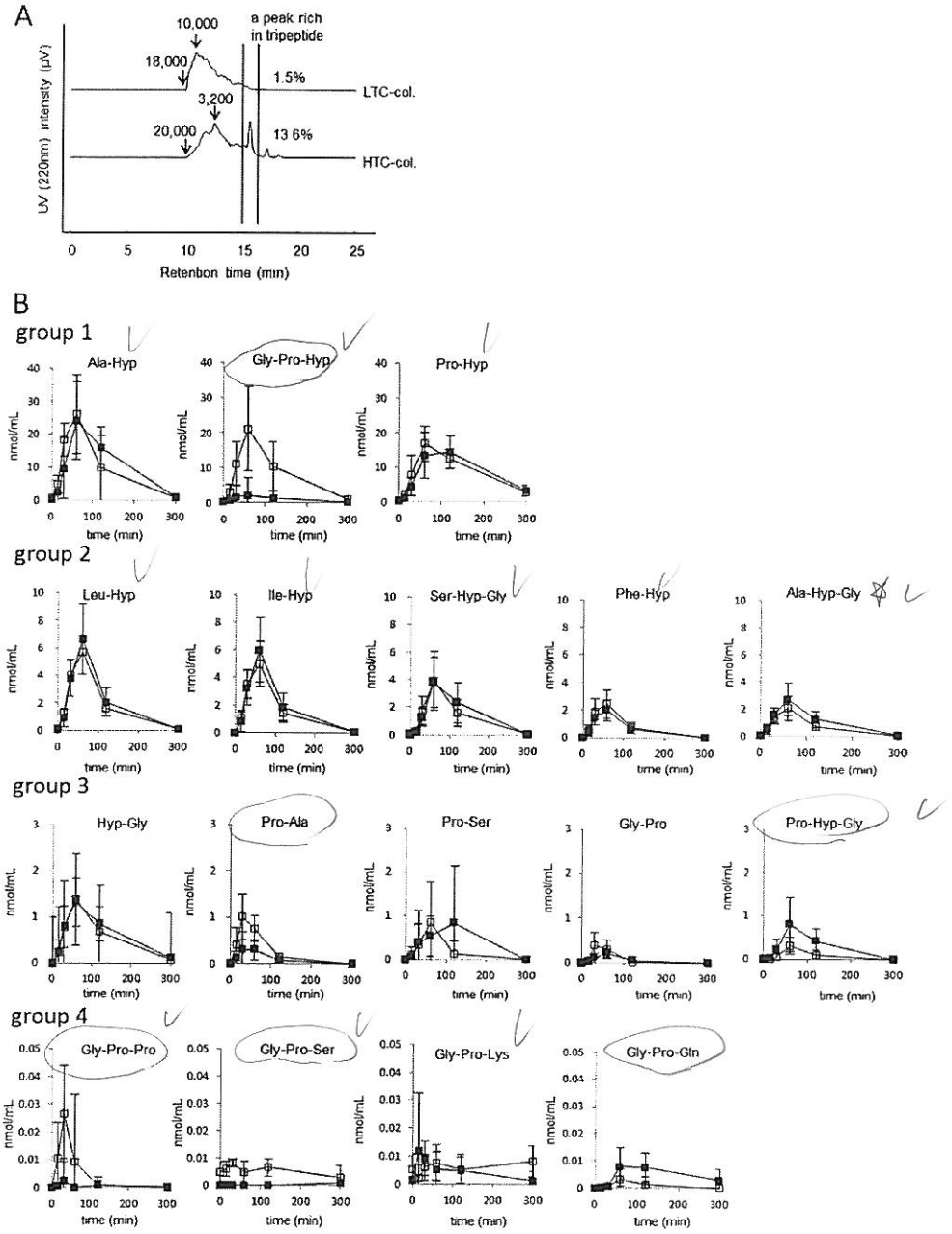


Figure 1. Molecular weight distribution of collagen hydrolysate detected by gel filtration chromatography (A). Plasma concentration of collagen-derived peptides after the oral ingestion of collagen hydrolysate in human study (B). Subjects ingested HTC-col (□) or LTC-col (■) at 300 mg per kg of body weight. Plasma concentration of peptides before and after ingestion was quantified using LC-MS/MS. Results were placed in descending order of $C_{max}$ and divided into four groups. Values are means ± SD, $n$ = 12.

1500−1800 Da. This is referred to as high tripeptide containing collagen hydrolysate (HTC-col). CPAH-S had an average molecular weight of 4500−5500 Da and is referred to as low tripeptide containing collagen hydrolysate (LTC-col). Synthetic peptides were purchased from Bachem (Bubendort, Switzerland).

**Gel Filtration Chromatography.** The molecular weight of collagen hydrolysate was evaluated by gel permeation chromatography HPLC using a Gulliver system (JASCO, Tokyo, Japan) equipped with a UV detector operating at 220 nm and a YMC-Pack Diol-60 column (500 × 8.0 mm i.d.; YMC, Kyoto, Japan). The mobile phase was 80% 0.1 M sodium phosphate buffer at pH 7.4 and 20% acetonitrile (Wako Chemicals, Osaka, Japan), and the flow rate was 1.0 mL/min.

**Human Study.** Four healthy men and eight healthy women were recruited to participate in this study. The average age of subjects was 31.5 ± 6.5 years. Exclusion criteria included the following: allergies to gelatin foods; liver, kidney, and gastrointestinal diseases; physical therapy or use of medication; pregnancy or during lactation; difficulty in take blood samples; blood donation within a month. This study was carried out with sufficient respect for the spirit of the Declaration of Helsinki and was approved by institutional review boards of FANCL Co. The study was undertaken using a single-blinded and crossover


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Table 1. Key Pharmacokinetic Parameters in Human Plasma after Oral Ingestion of Collagen Hydrolysates HTC-col and LTC-col[a]

| HTC-col | $C_{max}$[b] (nmol/mL) | AUC[c] (min·nmol/mL) | $t_{max}$[d] (min) | LTC-col | $C_{max}$ (nmol/mL) | AUC (min·nmol/mL) | $t_{max}$ (min) |
|---|---|---|---|---|---|---|---|
| 1 Ala-Hyp | 26.01 ± 11.76 | 2858.72 ± 1412.58 | 60 | 1 Ala-Hyp | 23.98 ± 11.99 | 3302.34 ± 1431.83 | 60 |
| 2 Gly-Pro-Hyp | 21.13 ± 12.07* | 2599.88 ± 1431.78* | 60 | 2 Pro-Hyp | 14.42 ± 4.76 | 2759.08 ± 825.12 | 120 |
| 3 Pro-Hyp | 16.84 ± 5.02 | 2696.92 ± 583.28 | 60 | 3 Leu-Hyp | 6.58 ± 2.54 | 647.97 ± 185.82 | 60 |
| 4 Leu-Hyp | 5.7 ± 2.39 | 565.6 ± 217.02 | 60 | 4 Ile-Hyp | 5.96 ± 2.33 | 580.97 ± 186.24 | 60 |
| 5 Ile-Hyp | 4.96 ± 2.00* | 489.99 ± 195.55 | 60 | 5 Ser-Hyp-Gly | 3.8 ± 1.83 | 484.071 ± 184.76 | 60 |
| 6 Ser-Hyp-Gly | 3.91 ± 2.16 | 408.36 ± 206.31 | 60 | 6 Ala-Hyp-Gly | 2.7 ± 1.18 | 321.14 ± 103.83 | 60 |
| 7 Phe-Hyp | 2.41 ± 1.01* | 243.11 ± 105.83 | 60 | 7 Gly-Pro-Hyp | 2.1 ± 5.09 | 323.17 ± 587.83 | 60 |
| 8 Ala-Hyp-Gly | 2.05 ± 0.91* | 222.68 ± 78.78* | 60 | 8 Phe-Hyp | 1.98 ± 0.77 | 203.11 ± 70.50 | 60 |
| 9 Hyp-Gly | 1.38 ± 0.72 | 171.43 ± 90.41 | 60 | 9 Hyp-Gly | 1.32 ± 0.52 | 191.44 ± 80.20 | 60 |
| 10 Pro-Ala | 1.01 ± 0.49* | 82.11 ± 29.15* | 30 | 10 Pro-Ser | 0.83 ± 1.31 | 132.42 ± 167.47 | 120 |
| 11 Pro-Ser | 0.85 ± 0.28 | 63.4 ± 61.46 | 60 | 11 Pro-Hyp-Gly | 0.8 ± 0.63 | 89.77 ± 64.83 | 60 |
| 12 Gly-Pro | 0.39 ± 0.29* | 25.81 ± 14.778* | 30 | 12 Pro-Ala | 0.31 ± 0.22 | 32.32 ± 25.02 | 60 |
| 13 Pro-Hyp-Gly | 0.31 ± 0.19* | 28.5 ± 15.17* | 60 | 13 Gly-Pro | 0.21 ± 0.10 | 17.82 ± 9.56 | 60 |
| 14 Gly-Pro-Pro | 0.03 ± 0.02* | 1.25 ± 1.448* | 30 | 14 Gly-Pro-Lys | 0.01 ± 0.02 | 1.27 ± 0.96 | 15 |
| 15 Gly-Pro-Ser | 0.01 ± 0.001* | 1.56 ± 0.78* | 30 | 15 Gly-Pro-Gln | 0.01 ± 0.007 | 1.5 ± 0.93 | 60 |
| 16 Gly-Pro-Lys | 0.01 ± 0.01* | 1.86 ± 0.86 | 300 | 16 Gly-Pro-Pro | 0.002 ± 0.008 | 0.18 ± 0.28 | 30 |
| 17 Gly-Pro-Gln | 0.003 ± 0.004* | 0.3 ± 0.48* | 60 | 17 Gly-Pro-Ser | 0.0009 ± 0.003 | 0.085 ± 0.29 | 300 |

[a]Values are the mean ± SD. An asterisk indicates significant difference compared to LTC-col by paired *t*-test; $p < 0.05$. [b]Maximum concentration. [c]Area under the curve. [d]Time at which the $C_{max}$ is observed.

study with no less than 7 days of washout period. After overnight fasting for 12 h, subjects orally ingested collagen hydrolysate HTC-col or LTC-col at a dose of 300 mg/kg of body weight with 20 w/v % water. Twelve milliliters of venous blood was collected in EDTA tubes just before and 15, 30, 60, 120, and 300 min after oral ingestion. The blood plasma was immediately separated by centrifugation and stored at −80 °C until analysis.

**Mouse Studies and Ethical Considerations.** All mouse studies were conducted under specific pathogen-free conditions. Mice were purchased from SLC (Shizouka, Japan) and acclimatized for a week before experiments and had free access to laboratory standard chow and water. All mice were divided to five per cage randomly. The experiments were performed at around 24 ± 3 °C temperature, with 55 ± 20% humidity and a12 h light/dark cycle. All protocols were approved by the animal ethics committee of FANCL Co.

**Mouse Study 1.** Five-week-old male BALB/c mice were randomly assigned into two groups. Groups were treated with either a low dose (900 mg/kg) or higher dose (1800 mg/kg) of HTC-col. Both groups were fasted for 16 h before the oral ingestion of collagen hydrolysate. After anesthetizing mice, blood was taken from the heart, before and 15, 30, 45, 60, 120, and 360 min after oral ingestion. We used five mice for each time point and dose. After blood collection, all mice were perfused with PBS to remove all blood, and the abdominal and dorsal skin was collected to measure peptide concentrations. The blood plasma and skin were stored at −80 °C until further analysis.

**Mouse Study 2.** Five-week-old male BALB/c mice were randomly assigned to orally ingest Gly-Pro-Hyp or Pro-Hyp (Bachem) at 0.2 mmol/kg. Blood was taken from the heart before and 10, 15, 30, 45, 60, and 120 min after oral ingestion, and the skin was collected after perfusion. We used five mice at each time point. The blood plasma and skin were stored at −80 °C until further analysis.

**Pretreatment of Samples for LC-MS/MS.** *Blood Plasma.* For the deproteinization of plasma, 3 volumes of ethanol was added to plasma and centrifuged at 400*g* for 10 min at 4 °C. Then the supernatant was used for quantification of peptide concentrations by LC-MS/MS.

*Skin.* After the skin had been crushed in liquid nitrogen, samples were sonicated in an equivalent weight of distilled water and centrifuged at 9000*g* for 5 min at 4 °C. As for the pellet, the same procedure was then repeated with 50% ethanol (Wako Chemicals, Osaka, Japan) and subsequently with 100% ethanol. The three supernatants were mixed and filtered through a 0.45 μm PVDF filter (Merck Millipore, Bedford, MA, USA). This filtrate was then subsequently injected into the LC-MS/MS system.

**LC-MS/MS Analysis.** Peptide concentrations in plasma and skin were quantified by LC-MS/MS. The LC was performed with an Acquity UPLC system with an Acquity UPLC HSS T3 column (Waters, Milford, MA, USA). Binary gradient elution was performed with 0.05% (v/v) heptafluorobutyric acid (Thermo Fisher Scientific, Waltham, MA, USA) containing 0.1% formic acid (Wako Chemicals) and acetonitrile. The gradient profile with the following proportions (v/v) of acetonitrile was applied (*t* (min), % acetonitrile): 0 min, 0%; 1 min, 0%; 4 min, 20%; 5 min, 30%; 5.2 min, 30%; 5.3 min, 100%; 6.3 min, 100%; 6.4 min, 0%; 9.5 min, 0%. The flow rate was 0.4 mL/min. Mass spectrometry was performed with electrospray ionization (ESI) in the positive mode, and the multiple-reaction monitoring system was used with an ion source temperature of 120 °C, a desolvation temperature of 400 °C, a desolvation gas flow of 600 L/h, a cone gas flow of 50 L/h, and a capillary voltage of 3 kV.

**Statistical Analyses.** Statistical analyses were performed using Microsoft Excel 2010 (Microsoft, Redmond, WA, USA). The $C_{max}$ and AUC of the HTC-col group and LTC-col group were compared by paired *t* test. $p < 0.05$ was considered to be statistically significant difference.

## RESULTS AND DISCUSSION

**Ingestion of HTC-col Increases Levels of Collagen-Derived Peptides in the Blood.** Previous studies have shown that ingestion of collagen hydrolysate leads to the transient incorporation of collagen-derived peptides such as Pro-Hyp, Phe-Hyp, Ala-Hyp, and Leu-Hyp into the blood.[11,24] These studies identified primarily dipeptides to be enriched rather than tripeptides. We hypothesized that the method of collagen hydrolysate preparation may influence the type of peptides incorporated into the blood. To test this, we first analyzed the composition of peptides present in two different types of collagen hydrolysate, HTC-col and LTC col, by gel filtration chromatography. These two preparations differ in their content of collagen-derived peptides, and on the basis of their molecular weight distribution, a peak rich in tripeptides was observed with HTC-col at approximately 13.6%, whereas no such peak was found in LTC-col, which had only 1.5% (Figure 1A). Therefore, by using these two preparations of collagen



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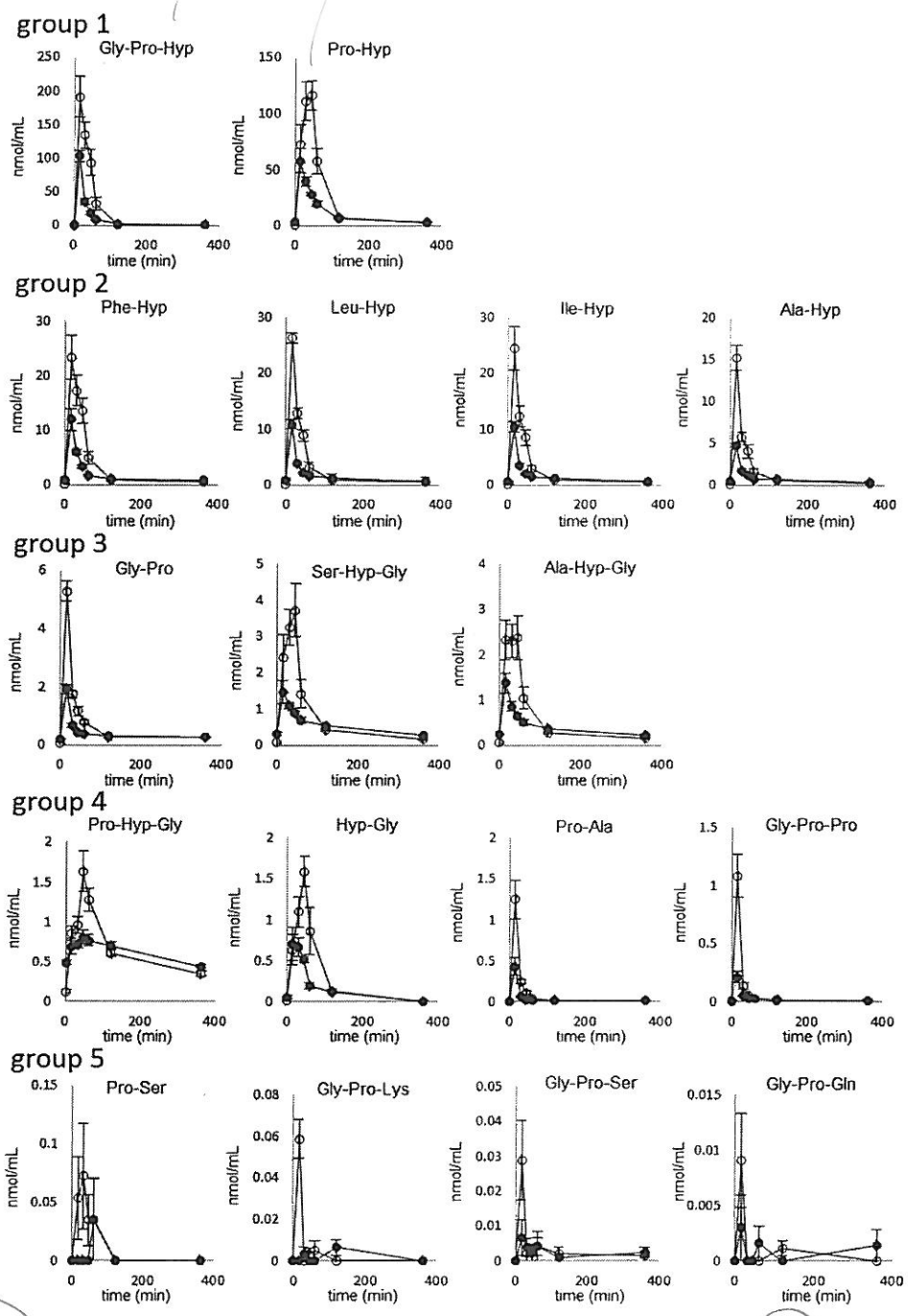


Figure 2. Plasma concentration of collagen-derived peptides after oral ingestion of collagen hydrolysate in the mouse study. Dose of HTC-col was 900 (●) or 1800 (○) mg/kg of body weight. Plasma concentration of peptides before and after ingestion was quantified using LC-MS/MS. Results were placed in descending order of $C_{max}$ and divided into five groups. Values are means ± SE, $n$ = 5.

hydrolysates, it would be possible to compare the specific contribution of tripeptides in the blood.

To examine the time-dependent changes of peptide concentrations in the blood after oral ingestion of collagen hydrolysate, 12 healthy individuals ingested either HTC-col or LTC-col (300 mg/kg body weight). Changes in collagen-derived peptide levels were evaluated by LC-MS/MS analysis over time. To select the peptide candidates for analysis, several criteria were used. First, peptides found present in HTC-col were chosen (data not shown). Thirteen peptides (Gly-Ala-Hyp, Gly-Leu-Hyp, Gly-Pro-Arg, Gly-Pro-Ala-, Gly-Pro-Gln, Gly-Pro-Hyp, Gly-Pro-Lys, Gly-Pro-Pro, Gly-Pro-Ser, Gly-Pro-Thr, Gly-Pro-Val, Pro-Pro, Pro-Ser) were detected in HTC-col. Second, among these 13 peptides, 11 peptides had the



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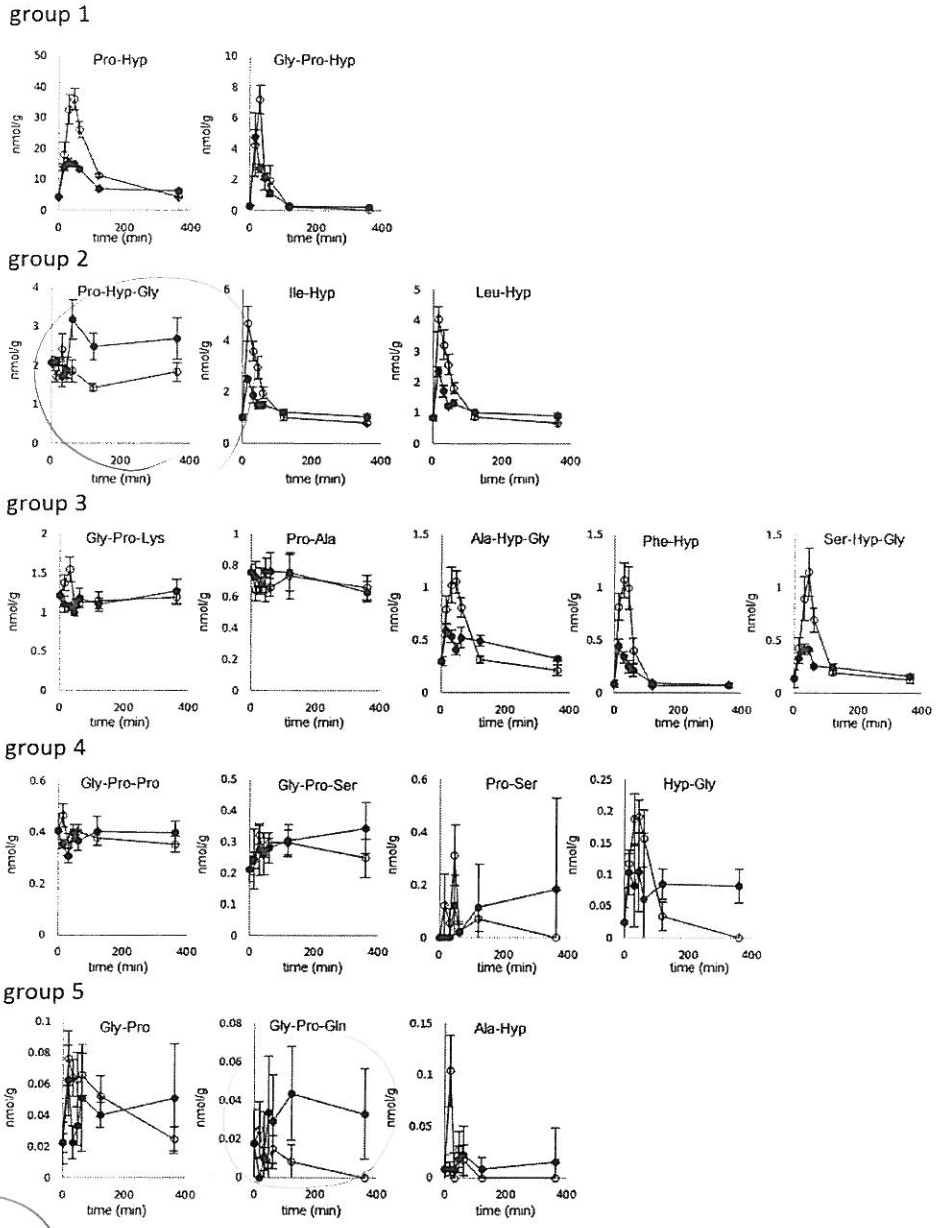


Figure 3. Skin concentration of collagen-derived peptides after oral ingestion of collagen hydrolysate in mouse study. Dose of HTC-col was 900 (●) or 1800 (○) mg/kg of body weight. Skin level of peptides before and after ingestion was quantified using LC-MS/MS. Results were placed in descending order of $C_{max}$ and devided into five groups. Values are means ± SE, $n = 5$.

sequence Gly-Pro-Y; therefore, Gly-Pro was also selected as a candidate. Third, peptides previously reported to be detected in the blood after the ingestion of collagen, namely, Ala-Hyp-Gly, Pro-Hyp-Gly, Ser-Hyp-Gly, Ala-Hyp, Hyp-Gly, Ile-Hyp, Leu-Hyp, Phe-Hyp, Pro-Hyp, and Pro-Ala, were also included. On the basis of these criteria, a total of 24 different peptides were measured in the blood. Of the 24 peptides, 17 were confirmed (Figure 1B). The $C_{max}$ (the maximum concentration) of the 17 peptides was plotted and categorized into four groups on the basis of their concentrations. Although previous papers described Pro-Hyp as the most abundant,[9−11] we found that Ala-Hyp and Gly-Pro-Hyp were more highly enriched. In fact, the peptide with the highest $C_{max}$ (HTC-col, 26.0 nmol/mL; and LTC-col, 24.0 nmol/mL) and area under the curve (AUC) (HTC-col, 2859 min·nmol/mL; and LTC-col, 3302 min·nmol/mL) was Ala-Hyp for both HTC-col and LTC-col. Interestingly, Gly-Pro-Hyp had the second highest $C_{max}$, but this was observed only upon ingestion of HTC-col (HTC-col, 21.1 nmol/mL; and LTC-col, 2.1 nmol/mL) (Table 1). The differences in amount of Gly-Pro-Hyp detected between HTC-col and LTC-col in terms of $C_{max}$ and AUC were approximately 10- and 8 fold, respectively. The majority of the top 10 peptides found (Table 1), with exception of Pro-Ala, had a <2-fold difference between HTC-col and LTC-col,



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Table 2. Key Pharmacokinetic Parameters in Mouse Plasma and Skin after Oral Ingestion of Collagen Hydrolysate

| 900 mg/kg plasma | $C_{max}$[a] (nmol/mL) | AUC (nmol·min/g) | $t_{max}$ (min) |
|---|---|---|---|
| 1 Gly-Pro-Hyp | 103.08 ± 8.40 | 2732.22 | 15 |
| 2 Pro-Hyp | 57.84 ± 10.64 | 2828.49 | 15 |
| 3 Phe-Hyp | 11.94 ± 1.94 | 366.39 | 15 |
| 4 Leu-Hyp | 10.85 ± 0.84 | 284.12 | 15 |
| 5 Ile-Hyp | 10.41 ± 0.97 | 319.19 | 15 |
| 6 Ala-Hyp | 4.75 ± 0.24 | 137.11 | 15 |
| 7 Gly-Pro | 1.93 ± 0.16 | 69.24 | 15 |
| 8 Ser-Hyp-Gly | 1.46 ± 0.32 | 75.86 | 15 |
| 9 Ala-Hyp-Gly | 1.37 ± 0.21 | 60.69 | 15 |
| 10 Pro-Hyp-Gly | 0.80 ± 0.09 | 47.22 | 45 |
| 11 Hyp-Gly | 0.70 ± 0.20 | 32.75 | 15 |
| 12 Pro-Ala | 0.42 ± 0.11 | 10.58 | 15 |
| 13 Gly-Pro-Pro | 0.21 ± 0.05 | 6.08 | 15 |
| 14 Pro-Ser | 0.03 ± 0.03 | 1.31 | 60 |
| 15 Gly-Pro-Lys | 0.01 ± 0.00 | 0.98 | 120 |
| 16 Gly-Pro-Ser | 0.01 ± 0.01 | 0.74 | 15 |
| 17 Gly-Pro-Gln | 0.00 ± 0.00 | 0.27 | 15 |
| **900 mg/kg skin** | **$C_{max}$ (nmol/mL)** | **AUC (nmol·min/g)** | **$t_{max}$ (min)** |
| 1 Pro-Hyp | 15.34 ± 1.53 | 1425.14 | 30 |
| 2 Gly-Pro-Hyp | 4.72 ± 0.46 | 161.62 | 15 |
| 3 Pro-Hyp-Gly | 3.17 ± 0.51 | 175.35 | 60 |
| 4 Ile-Hyp | 2.50 ± 0.11 | 92.23 | 15 |
| 5 Leu-Hyp | 2.33 ± 0.15 | 99.50 | 15 |
| 6 Gly-Pro-Lys | 1.27 ± 0.15 | 6.56 | 360 |
| 7 Pro-Ala | 0.77 ± 0.12 | 0.42 | 60 |
| 8 Ala-Hyp-Gly | 0.59 ± 0.06 | 50.64 | 15 |
| 9 Phe-Hyp | 0.44 ± 0.07 | 17.71 | 15 |
| 10 Ser-Hyp-Gly | 0.42 ± 0.05 | 35.52 | 30 |
| 11 Gly-Pro-Pro | 0.41 ± 0.07 | 0.00 | 0 |
| 12 Gly-Pro-Ser | 0.35 ± 0.04 | 33.85 | 360 |
| 13 Pro-Ser | 0.18 ± 0.15 | 42.18 | 360 |
| 14 Hyp-Gly | 0.11 ± 0.03 | 20.54 | 45 |
| 15 Gly-Pro | 0.06 ± 0.01 | 7.94 | 15 |
| 16 Gly-Pro-Gln | 0.04 ± 0.01 | 6.40 | 120 |
| 17 Ala-Hyp | 0.02 ± 0.01 | 1.50 | 60 |

| 1800 mg/kg plasma | $C_{max}$ (nmol/mL) | AUC (nmol·min/g) | $t_{max}$ (min) |
|---|---|---|---|
| 1 Gly-Pro-Hyp | 191.42 ± 30.46 | 7802.11 | 15 |
| 2 Pro-Hyp | 72.73 ± 17.47 | 7790.06 | 15 |
| 3 Leu-Hyp | 26.27 ± 3.85 | 959.36 | 15 |
| 4 Ile-Hyp | 24.38 ± 3.88 | 913.14 | 15 |
| 5 Phe-Hyp | 23.39 ± 4.01 | 1132.78 | 15 |
| 6 Ala-Hyp | 15.15 ± 1.52 | 521.49 | 15 |
| 7 Gly-Pro | 5.28 ± 0.34 | 206.72 | 15 |
| 8 Ser-Hyp-Gly | 3.72 ± 0.74 | 250.12 | 45 |
| 9 Ala-Hyp-Gly | 2.38 ± 0.49 | 182.51 | 45 |
| 10 Pro-Hyp-Gly | 1.63 ± 0.25 | 187.66 | 45 |
| 11 Hyp-Gly | 1.58 ± 0.18 | 94.94 | 45 |
| 12 Pro-Ala | 1.25 ± 0.24 | 27.32 | 15 |
| 13 Gly-Pro-Pro | 1.08 ± 0.19 | 20.51 | 15 |
| 14 Pro-Ser | 0.07 ± 0.04 | 3.74 | 30 |
| 15 Gly-Pro-Ser | 0.03 ± 0.01 | 1.10 | 15 |
| 16 Gly-Pro-Lys | 0.06 ± 0.01 | 1.10 | 15 |
| 17 Gly-Pro-Gln | 0.01 ± 0.00 | 0.30 | 15 |
| **1800 mg/kg skin** | **$C_{max}$ (nmol/mL)** | **AUC (nmol·min/g)** | **$t_{max}$ (min)** |
| 1 Pro-Hyp | 35.81 ± 3.50 | 2943.98 | 45 |
| 2 Gly-Pro-Hyp | 7.17 ± 0.91 | 248.49 | 30 |
| 3 Ile-Hyp | 4.64 ± 0.68 | 157.19 | 15 |
| 4 Leu-Hyp | 4.02 ± 0.39 | 151.89 | 15 |
| 5 Ser-Hyp-Gly | 1.15 ± 0.23 | 60.58 | 45 |
| 6 Pro-Hyp-Gly | 2.39 ± 0.39 | 4.86 | 30 |
| 7 Gly-Pro-Lys | 1.54 ± 0.16 | 7.43 | 30 |
| 8 Phe-Hyp | 1.07 ± 0.17 | 51.19 | 30 |
| 9 Ala-Hyp-Gly | 1.05 ± 0.11 | 51.81 | 45 |
| 10 Pro-Ala | 0.76 ± 0.05 | 0.00 | 0 |
| 11 Gly-Pro-Pro | 0.47 ± 0.05 | 0.92 | 15 |
| 12 Gly-Pro-Ser | 0.32 ± 0.03 | 22.90 | 30 |
| 13 Pro-Ser | 0.31 ± 0.12 | 18.83 | 45 |
| 14 Hyp-Gly | 0.19 ± 0.03 | 12.80 | 45 |
| 15 Gly-Pro | 0.08 ± 0.02 | 8.50 | 15 |
| 16 Ala-Hyp | 0.03 ± 0.02 | 2.07 | 45 |
| 17 Gly-Pro-Gln | 0.02 ± 0.01 | 0.11 | 15 |

[a]Values are the mean ± SE.

indicating that Gly-Pro-Hyp has a distinct biokinetic profile. These results demonstrate that the ingestion of HTC-col, which contains high concentrations of Gly-Pro-Hyp, leads to the transiently high incorporation of GLy-Pro-Hyp in plasma. Furthermore, as this was not observed with LTC-col, this implies that the method of collagen hydrolysate preparation is critical and that this would likely influence the effects of collagen hydrolysate on the body.

**Administration of HTC-col in Mice Increases the Incorporation of Gly-Pro-Hyp in the Blood.** To determine whether the collagen-derived peptides observed in the blood are incorporated into the skin, we used an in vivo mouse model. The levels of the 17 peptides that were detected in humans (Figure 1B) were analyzed in the blood and skin of mice after oral ingestion of HTC-col. After the administration of HTC-col at a dose of either 900 or 1800 mg/kg, a transient increase in collagen-derived peptides was detected in a dose-dependent manner in both plasma (Figure 2) and skin (Figure 3). The $C_{max}$ values at 900 mg/kg of the peptides were plotted and categorized into five groups according to their concentrations for the blood and skin. Ala-Hyp was the most abundant peptide detected in human blood (Figure 1B), and from the $C_{max}$ and AUC, it was observed in the second highest group in mice (Figure 2, group 2). Surprisingly, however, the concentration of Ala-Hyp incorporated into the skin was very low (Figure 3, group 5), suggesting that the skin may have a peptide specificity for efficient incorporation or, alternatively, peptides in the blood may be further hydrolyzed. Instead, the peptide detected with the highest $C_{max}$ in the blood was Gly-Pro-Hyp (103 nmol/mL at 900 mg/kg and 191 nmol/mL at 1800 mg/kg) (Table 2). The AUC of Gly-Pro-Hyp in the blood, when compared to the other peptides, was found to be the highest at 1800 mg/kg (7802 min·nmol/mL), and it was the second highest at 900 mg/kg (2732 min·nmol/mL). Interestingly, peptide concentrations in the skin did not directly correlate with those of the blood. In particular, whereas the $C_{max}$ of Gly-Pro-Hyp in plasma was 1.78-fold (900 mg/kg) or 2.63-fold (1800 mg/kg) higher than that of Pro-Hyp, in the skin, the $C_{max}$ of Gly-Pro-Hyp was 0.31- or 0.20-fold lower than that of Pro-Hyp. A possible explanation for these discrepancies is that some tripeptides may be converted into dipeptides, such that the Pro-Hyp detected in the skin may actually be derived from Gly-Pro-Hyp in the blood.

Consistent with the expected kinetics of peptides ingested, we found that the $t_{max}$ (the time at which the $C_{max}$ is observed), for both Gly-Pro-Hyp and Pro-Hyp, was shorter in the blood



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(15 min for both Gly-Pro-Hyp and Pro-Hyp) than in the skin (30 min for Gly-Pro-Hyp; 45 min for Pro-Hyp). Therefore, this further supports that the peptides detected in the skin reflect those absorbed into the skin tissue from the blood. Taken together, we conclude that oral ingestion of collagen hydrolysate, HTC-col, results in the incorporation of a set of collagen-derived peptides such as Gly-Pro-Hyp, Pro-Hyp-Gly, and Pro-Hyp into the skin.

**Administration of Pure Gly-Pro-Hyp or Pro-Hyp Peptides Causes an Increase in Accumulation of Pro-Hyp in the Skin.** To determine the cause for differences observed in the blood and skin, we investigated the effects of administering either pure Gly-Pro-Hyp or Pro-Hyp (0.2 mmol/kg body weight) in mice. Measuring the peptide levels in plasma and skin, after the ingestion of Gly-Pro-Hyp, not only had the plasma concentration of Gly-Pro-Hyp increased but also that of Pro-Hyp was higher (Figure 4A). Consistent with this result, we observed a similar trend in the skin (Figure 4B). Additionally, when Pro-Hyp was administered, the AUC and $C_{max}$ were only 0.997- and 1.09-fold higher than when Gly-Pro-Hyp was administered (Table 3), respectively. These results indicate that the intake of Gly-Pro-Hyp causes the increase in both Gly-Pro-Hyp and Pro-Hyp in the plasma and the skin. This suggests, at least in part, that the Pro-Hyp detected in the skin is derived from Gly-Pro-Hyp. Because Pro-Hyp was detected in the blood 15 min after the administration of pure Gly-Pro-Hyp, a fraction of Gly-Pro-Hyp may be hydrolyzed within 15 min. Furthermore, this implies that Gly-Pro-Hyp is likely to be present in the skin only transiently. This result is also consistent with a previous study that showed Gly-Pro-Hyp is partially hydrolyzed on the apical membrane[25] and transported into the cells via the peptide transporter PETP1.[26,27]

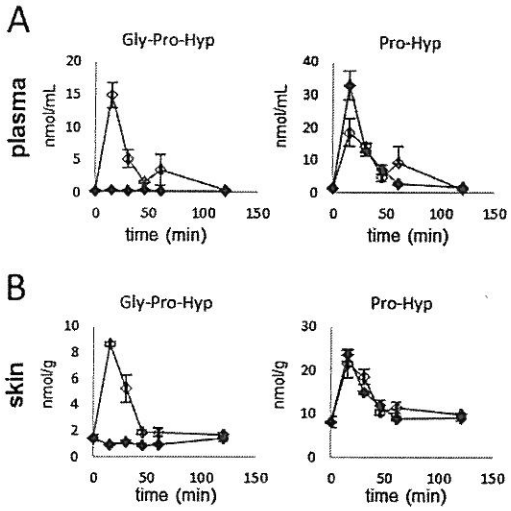


Figure 4. Absorption and degradation of pure Gly-Pro-Hyp and Pro-Hyp in the mouse study. Gly-Pro-Hyp (◇) or Pro-Hyp (◆) was ingested at 0.2 mmol/kg of body weight. Peptide concentrations of plasma (A) and skin (B) before and after ingestion were quantified using LC-MS/MS. Values are means ± SE, $n$ = 5.

Previous studies have shown that Pro-Hyp has beneficial effects by stimulating cell proliferation, enhancing hyaluronic acid synthesis,[16,17] and promoting cell migration[18] when added to skin-derived cells in vitro. In the present study, we have shown the transient increase in Gly-Pro-Hyp levels in the blood of both humans and mice by the oral administration of HTC-col, which has high concentrations of tripeptides. This tripeptide is then further hydrolyzed to Pro-Hyp in the blood and skin. We found that not only Pro-Hyp but also other collagen-derived peptides were transported to the skin by the ingestion of HTC-col. On the basis of our data, we believe that it is unlikely that Pro-Hyp is the only peptide in collagen hydrolysate that has beneficial effects. Furthermore, as synthesizing pure peptides would be highly cost inefficient to manufacture as a dietary supplement, our data demonstrate not only that HTC-col would be a cheaper alternative but also that, as it contains other collagen-derived peptides, it is more suitable as a food supplement and potentially for skin healthcare.

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### Notes

The authors declare no competing financial interest.

Table 3. Key Pharmacokinetic Parameters in Mouse Plasma and Skin after Oral Ingestion of Gly-Pro-Hyp or Pro-Hyp

| administered substance | measurement target | | $C_{max}$[a] (nmol/mL) | AUC (nmol·min/g) |
|---|---|---|---|---|
| Gly-Pro-Hyp | plasma | Gly-Pro-Hyp | 14.78 ± 2.00 | 431.59 |
| | | Pro-Hyp | 18.35 ± 4.33 | 796.03 |
| | skin | Gly-Pro-Hyp | 8.62 ± 0.15 | 193.86 |
| | | Pro-Hyp | 21.39 ± 3.13 | 561.06 |
| Pro-Hyp | plasma | Gly-Pro-Hyp | 0.23 ± 0.05 | 1.09 |
| | | Pro-Hyp | 32.75 ± 4.30 | 794.02 |
| | skin | Gly-Pro-Hyp | 1.42 ± 0.21 | 0.00 |
| | | Pro-Hyp | 23.41 ± 1.49 | 439.75 |

[a]Values are the mean ± SE.



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## ABBREVIATIONS USED

HTC-col, high tripeptide containing collagen hydrolysate; LTC-col, low tripeptide containing collagen hydrolysate; AUC, area under the curve; $C_{max}$, maximum concentration; $t_{max}$, time at which the $C_{max}$ is observed

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